Abnormal Lignin in a Loblolly Pine Mutant

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Novel lignin is formed in a mutant loblolly pine (Pinus taeda L.) severely depleted in cinnamyl alcohol dehydrogenase (E.C. 1.1.1.195) which converts coniferaldehyde to coniferyl alcohol, the primary lignin precursor in pines. Dihydroconiferyl alcohol, a monomer not normally associated with the lignin biosynthetic pathway, is the major component of the mutant's lignin, accounting for ~30% (vs. ~3% in normal pine) of the units. The level of aldehydes, including new 2-methoxybenzaldehydes, is also elevated. The mutant pines grew normally indicating that, even within a species, extensive variations in lignin composition need not disrupt lignin's essential functions.

Lignins are complex phenolic plant polymers essential for mechanical support, defense, and water transport in vascular terrestrial plants (1, 2). They are usually derived from three hydroxycinnamyl alcohol precursors **2a-c** in varying proportions, Figure 1. In gymnosperms, for example pine and other conifers, lignin is polymerized from only two of the three monomers, *p*-coumaryl alcohol **2a** and coniferyl alcohol **2b**, with coniferyl alcohol being predominant (~90%). *p*-Coumaryl alcohol-derived subunit levels are elevated in compression wood which forms during mechanical or gravitational stress and in wood knots (3). In woody angiosperms, lignin is derived from coniferyl alcohol **2b** and sinapyl alcohol **2c** in roughly equal proportions. It is increasingly recognized that precursors and derivatives of hydroxycinnamyl alcohols also contribute to the lignin structure. For example, acetylated monolignols (hydroxycinnamyl acetates) have been implicated in kenaf (*Hibiscus cannabinus*) (4) and woody angiosperms (5), and *p*-coumarate esters are found in all grass lignins implicating hydroxycinnamyl *p*-coumarates as precursors (6, 7). Low levels (~5%) of cinnamaldehydes and benzaldehydes are found in all isolated lignins and are responsible for the bright crimson staining of lignified tissues by phloroglucinol/HCl (8).

Removal of lignin from wood and plant fibers is the basis of chemical pulping to produce diverse pulp and paper products. Genetic engineering of the lignin biosynthetic pathway to lower lignin concentration or construct lignins more amenable to extraction is an active area of current research (9). However, several mutations have been identified and characterized that affect the lignin biosynthetic pathway (10). In maize (*Zea mays*) and related grasses, mutants characterized by a brown midrib (*bm* or *bmr*) have modified lignin (11). The *bm* phenotype can result from changes affecting cinnamyl alcohol dehydrogenase (CAD) (for example *bm1* of maize) (12, 13), O-methyl transferase (OMT) (for example *bm3* of maize) (12, 13), or both CAD and OMT (for example *bmr6* of sorghum, *Sorghum bicolor*) (14). Mutations in two other maize genes also lead to brown midrib phenotypes, but the products of these genes remain unknown. A mutation in the gene encoding ferulate-5-hydroxylase has been identified in *Arabidopsis thaliana*, but it does not result in a brown midrib phenotype (15). No lignin mutants have been previously identified in woody plants.

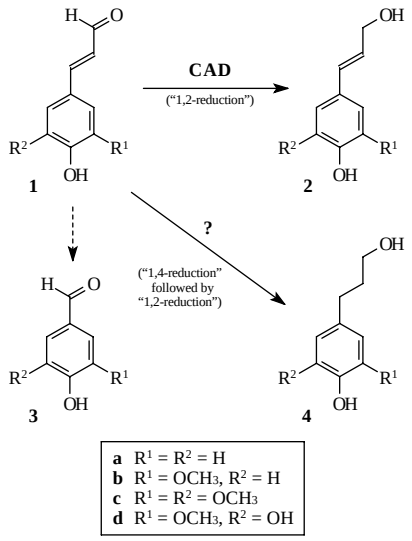

Figure 1. Some precursors and products involved in the lignin biosynthetic pathway. The normal lignin monomers are the *p*-hydroxycinnamyl alcohols **2**; *p*-coumaryl alcohol **2a**, coniferyl alcohol **2b** and sinapyl alcohol **2c**. Coniferaldehyde **1b** is normally reduced regioselectively to produce coniferyl alcohol **2b**. When CAD activity is depressed, coniferaldehyde **2b** accumulates and could polymerize or co-polymerize into lignin. Dihydroconiferyl alcohol **4b**, observed previously only as a minor component of softwood lignins, is presumed to derive from coniferaldehyde **1b** via a 1,4- followed by a 1,2-reduction. However, no mechanism for this conversion has been reported. *p*-Coumaryl alcohol **2a** is readily derived from its aldehyde **1a** in the mutant, implying that different CAD enzymes are involved for **1a**→**2a** *vs.* **1b**→**2b**.

CAD catalyses the last step of the lignin precursor biosynthetic pathway (Figure 1), reduction of hydroxycinnamaldehydes **1** to hydroxycinnamyl alcohols **2** (the conventional lignin monomers or monolignols) (16). A reduction in CAD activity might lead to accumulation of hydroxycinnamaldehydes **1** which could copolymerize with normal lignin monomers. Transgenic plants, suppressed in the synthesis of CAD (9, 17) sometimes have red-brown xylem tissue, resembling that of grass brown midrib mutants. Such plants have increased aldehyde levels, although little of the aldehyde may actually be incorporated into the lignin (9, 17). The molecular basis for the color has not been established, but higher order polymers of coniferaldehyde **1b**, synthesized *in vitro*, have a wine red color (18).

Here we report that a viable loblolly pine, homozygous for the mutant *cad-n1* allele (19), incorporates novel monomers into its lignin in response to a CAD deficiency. The lignin structural changes were extensive and not predicted by the current view of the lignin biosynthetic pathway. The wood of this mutant is brown-red (Figure 2), similar to the color of the xylem in *brown midrib* mutants (11) and transgenic plants suppressed in lignin biosynthetic enzyme activity (9, 17). The *cad-n1* allele is inherited as a Mendelian recessive gene that maps to the same genomic region as the *cad* locus. The *cad-n1* allele was identified in a well characterized loblolly pine heterozygous genotype (clone 7-56). In homozygous *cad-n1* plants, CAD activity is 1% or less of wild type, and relative abundance of *cad* mRNA transcript is greatly decreased. In mutant plants, free coniferaldehyde **1b**

Figure 2. Left: Wood chips from normal wood and a homozygous mutant with reduced CAD activity showing the brown wood phenotype. The mutant wood was obtained from a field test containing progeny from a cross between two half sib loblolly pines each heterozygous for the mutant cad gene. Right: Immediately after debarking, 2-year old trees. The *cad-n1* mutant is readily identified by the red-brown color of its wood.

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Table 1. Estimates of subunit compositions (from quantitative ^{13}C-NMR and DFRC-method data) of the normal and mutant pine isolated lignins. **2a** = *p*-coumaryl alcohol units; **2b** = coniferyl alcohol units; **1** = cinnamaldehyde units (*p*-hydroxycinnamaldehyde and coniferaldehyde units are not distinguished); **3** = benzaldehyde units (*p*-hydroxybenzaldehyde and vanillin units are not distinguished); ***** aldehydes at ~188 ppm in ^{13}C-NMR spectra are identified as 2-methoxybenzaldehydes **6** (22) from unknown sources — the other substituents on the aromatic ring are unknown; **4b** = dihydroconiferyl alcohol (+ traces of dihydro-*p*-coumaryl alcohol **4a**), the major component of the *cad-n1* mutant lignin.

Lignin	1	2a	2b	3	4b	*
cad-n1-mutant	15	10	15	15	30	15
cad-normal	7	10	73	7	3	trace

(the CAD substrate) accumulates to a high level. Unlike transgenic plants suppressed in CAD, *cad-n1* mutant seedlings have decreased lignin content (19).

Milled wood lignins (20) were isolated for NMR analysis from the wood of a 12 year old CAD-deficient mutant and a normal sibling from the same cross (Figure 2). An estimate of the subunit composition of this unusual lignin fraction, based on quantitative NMR and other analytical data, is given in Table 1.

NMR spectra show that both coniferaldehyde **5** and vanillin **7** (21) endgroups are present in the lignin of the pine mutant as may be expected from the suppression of CAD. Wood from the mutant also had a higher extractable aldehydes content (19). The HMQC-TOCSY spectrum reveals the sidechain coupling network with protons 7, 8, and 9 correlating with the aldehyde carbonyl carbon, C-9 in **5** and the simple 1-bond correlation between C-7 and H-7 in **7** (Figure 3). However, such components are also present in milled wood lignins from normal loblolly pine (Figure 3). From quantitative NMR, these aldehydes each account for ~15% of mutant lignin units and ~7% in the normal pine lignin (Table 1). More striking are 2-methoxybenzaldehyde components **6** (22), the peaks at ~188 ppm, that are greatly enhanced in the mutant (Figure 3). The source of these previously unreported 2-methoxybenzaldehydes in lignins is unknown. Lignins from both the normal and mutant trees contained higher than normal concentrations of *p*-coumaryl alcohol units due to the preponderance of knots.

Dihydroconiferyl alcohol units **8** are present and predominant. The HMQC-TOCSY experiment (23) (Figure 4) identified the coupling network for the aryl propanol sidechain (red and orange contours) that are consistent with model compound data (24). Products **8a**,**b** (red) representing hetero-coupling of dihydroconiferyl alcohol with a conventional lignin monomer/oligomer as well as dibenzodioxocins **8c** (orange) from initial 5–5-homo-coupling of dihydroconiferyl alcohol monomers are present in roughly equal amounts reinforcing the claim that dihydroconiferyl alcohol is a major monomer during lignification.

Dihydroconiferyl alcohol products are seen in synthetic lignins that are prepared from impure monomers. The best syntheses of hydroxycinnamyl alcohols **2** (25) from hydroxycinnamate esters or hydroxycinnamaldehydes **1**, still produce small amounts of 1,4-reduction products **4**. Purification of coniferyl alcohol is difficult because dihydroconiferyl alcohol co-crystalizes with it. A synthetic lignin prepared (26) from coniferyl alcohol **2b** containing a few percent dihydroconiferyl alcohol **4b** provides a convenient model for the lignin in the pine mutant. Its HMQC-TOCSY spectrum (Figure 4c) shows the same dihydroconiferyl alcohol sidechain signals as in the pine lignins. A parallel between the lignins isolated from the mutant tree and hydride reduction product synthetic lignins is apparent. A small amount of the initial dihydroconiferyl alcohol homo-coupling product **8c** (orange) is seen in the spectrum of the synthetic lignin (Figure 4c) — the saturated compound is quickly and efficiently polymerized via radical processes. Normal softwood lignins contain small amounts of dihydroconiferyl alcohol units (Figure 4b). The source of these subunits is unknown. Although dihydroconiferyl alcohol and its glucoside have been found in young plant tissues including pine (27), and may function as growth factors (28), they are not considered part of the normal lignin biosynthetic pathway.

CAD normally effects a regioselective "1,2-reduction" (at C-9) of coniferaldehyde **1b** to produce coniferyl alcohol **2b**. Our results suggest that the loss of CAD activity activates or upregulates pathways based on "1-4 reduction" (at C-7) and subsequent 1,2-reduction during lignin formation to produce the dihydroconiferyl alcohol monomer **4b** (Figure 1). Analogously, synthetic preparation of coniferyl alcohol versus dihydroconiferyl alcohol can be selected by hydride reactions with 1,2- *vs*. 1,4-regiochemistry, Figure 1 (25). An alternative possibility is that a small structural change in the enzyme (for example, a disulfide bridge) affecting the active site of the CAD enzyme might be enough to provide the "hydride" equivalent to the 7-carbon site. This possibility is unlikely and an alternative enzymatic activity is probably required because the relative abundance of steady state *cad* mRNA transcripts is greatly decreased in the mutant and the amount of CAD enzyme activity is reduced to ≤1% of wild type (19). If the biochemical reduction is not totally regioselective, the small amounts of **4b** producing the dihydroconiferyl units **8** seen in normal pine lignins could be explained but this rationale would not allow production of **4b** in such major proportions without a significant shift in enzyme activity or without enhanced activity of an alternate enzyme. At least one new enzyme would be required to explain these results. It is also possible that coniferaldehyde is not the precursor to dihydroconiferyl alcohol, and that its synthesis is up-regulated from other sources in the plant.

The amount of subunits derived from *p*-coumaryl alcohol **2a** in the mutant is unchanged (Table 1), while the amount of coniferyl alcohol subunits **2b** is greatly reduced (29). These results imply that the formation of *p*-coumaryl alcohol **2a** utilizes an independent mechanism such as an additional enzyme with "1,2-reductase activity" specific for *p*-hydroxycinnamaldehyde **1a**. Furthermore, few dihydro-*p*-coumaryl alcohol **4a** units were detected (29). The 1,4-reductase activity proposed for the formation of dihydroconiferyl alcohol is therefore equally specific for coniferaldehyde **1b**.

Incorporation of novel monomers into lignin is inconsistent with the high level of enzymatic specificity recently extended to lignin formation from observations of specificity in lignan biosynthesis (30). Independence from rigid enzymatic control is further supported by other examples of incorporation of non-traditional monomers into lignins: (i) ferulates and diferulates actively incorporate into lignins of grasses, effecting significant lignin-polysaccharide cross-linking (31); (ii) acylated monomers are implicated in a variety of species (4-7); (iii) 5-hydroxyconiferyl alcohol **2d** derived subunits are readily assimilated into a lignin polymer in OMT-deficient plants that have a reduced ability to produce sinapyl alcohol (32).

Well characterized differences in lignin subunit composition have long been known between major taxonomic groups of higher of plants, for example between lignins of hardwood and softwood trees (33). However, the narrow range of variation in lignin compositions within groups (10) has suggested structural constraints imposed for vascular function and support. The ability of this pine mutant to produce a functional lignin polymer from unexpected subunits extends the limit of "metabolic plasticity" for the formation of lignin, within an individual species. Concepts of lignin function based on the previous range of lignin compositions must now be reexamined in view of the unusual structure and composition of lignin in this mutant pine. A greater understanding of these processes should increase our opportunities to modify lignin content or composition through genetic engineering.

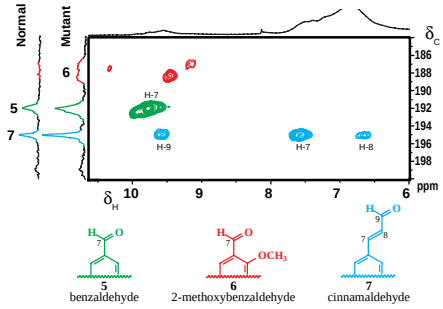

Figure 3. Aldehyde carbonyl group correlations from the HMQC-TOCSY (23) spectrum of lignin from the *cad-n1* mutant plant showing the presence of cinnamaldehyde (with correlations to the three sidechain protons) and benzaldehyde (single correlations) units in the lignin. The normal pine lignin carbon section is shown to the left. The ^{13}C-spectra shown on projections to the left of the figure are normalized to the same methoxyl level; cinnamaldehyde **7** and benzaldehyde **5** signals are approximately twice as abundant in the mutant. The higher field aldehydes dramatically increased in the mutant have now been identified as 2-methoxybenzaldehydes **6** (22); their source is unknown.

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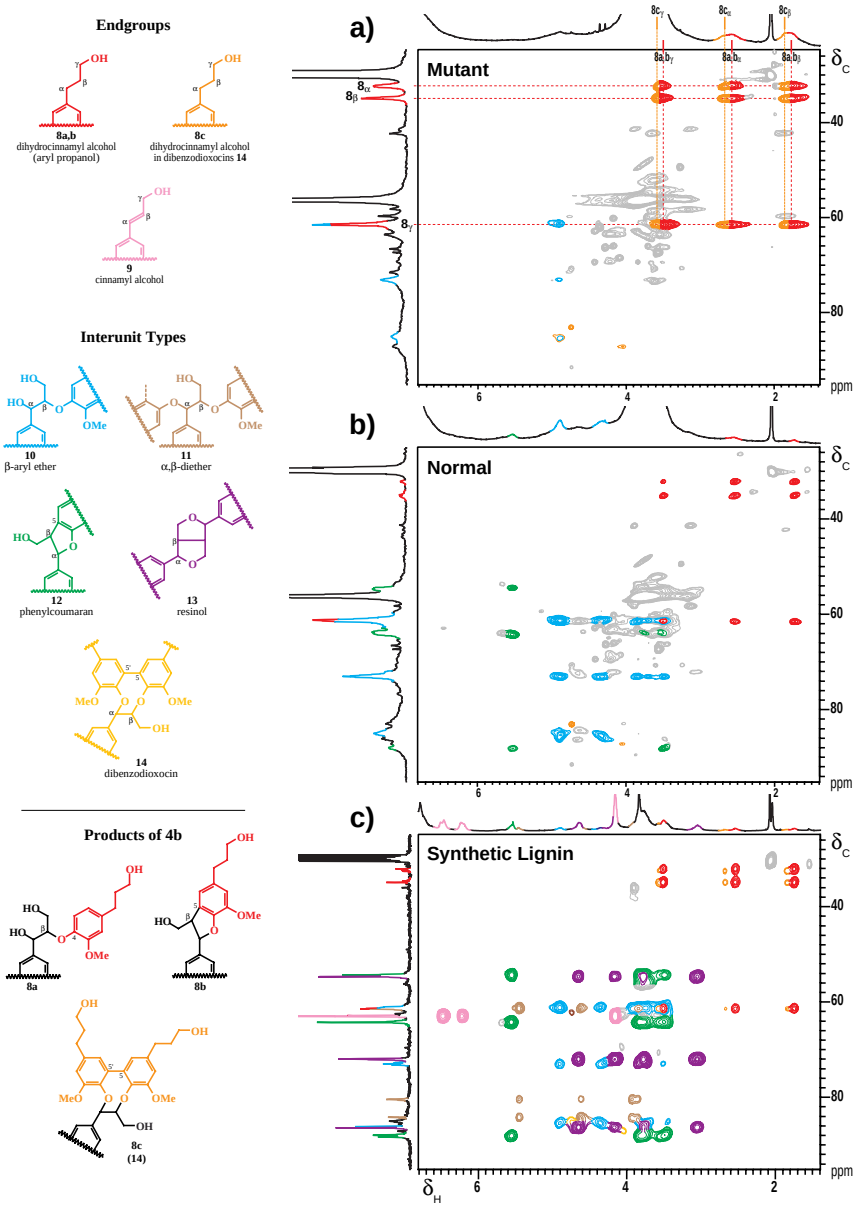

Figure 4. Regions of the HMQC-TOCSY spectra (23) of milled wood lignins from a) the pine *cad-n1* mutant, b) from a *cad*-normal wood, and c) from a synthetic lignin (26). Structure assignments are most easily seen in spectrum 4c from the synthetic lignin which derived from coniferyl alcohol **2b** containing ~2% dihydroconiferyl alcohol **4b** (26). Although synthetic lignins of this type have quite different substructure ratios from plant lignins, they contain all of the structural units and are valuable for spectral assignment. Thus in Figure 4c, β-aryl ether units **10**, α,β-diaryl ethers **11** (scarce in plant lignins), phenylcoumarans **12**, and resinols **13** are readily identified, along with coniferyl alcohol endgroups **9**, and the dihydroconiferyl alcohol units **8** (and the aldehyde units **5** and **7** in Figure 3). Red and orange colored contours show the unambiguously identified components **8** arising from dihydroconiferyl alcohol monomers **4**. NMR provides a convenient distinction between products of hetero-coupling of **4** with conventional lignin monomers/oligomers to give **8a**,**b** (red) and those from initial 5–5-homo-coupling of dihydroconiferyl alcohol monomers to give **8c** (orange). Both are equally represented in the *cad-n1* mutant, whereas the normal pine has only the higher field component, and the synthetic lignin has a trace of the lower field component. NMR data from cross-coupled dimeric models for 4-O-β structures **8a** and 5-β/4-O-α (phenylcoumaran) structures **8b** and the dibenzodioxocin **8c** coincide with the lignin data observed here (24). In the CAD mutant, dihydroconiferyl units are dominant, displacing much of the intensity from the normal coniferyl alcohol-derived region. Some of the minor units can be seen in the pine samples when looking at lower contour levels (not shown). The normally predominant β-aryl ether (blue) and phenylcoumaran (green) components, Figure 4b, are severely reduced in the *cad-n1* mutant, with only some β-ether peaks being observable at comparable contour levels — these may also arise from *p*-coumaryl alcohol (in addition to coniferyl alcohol). Grey contours are from intense methoxyl signals, carbohydrate impurities, and other lignin structures not discussed in this paper.

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13. Enzymes are denoted by capital letters: CAD = cinnamyl alcohol dehydrogenase (E.C. 1.1.1.195), OMT = O-Methyl transferase (E.C. 2.1.1.6), F5H = ferulate 5-hydroxylase (E.C. not available). Gene loci are denoted using lower case italics (for example *cad*), and the CAD-deficient mutant is denoted *cad-n1*.
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20. "Milled wood lignin" [A. Björkman, *Nature* **174**, 1057-1058 (1954)] were isolated essentially by methods previously described (7). Wood was first ground in a Wiley mill (1 mm screen), and soluble phenolics, carbohydrates, and other components were removed by successive extractions with diethyl ether, acetone, methanol, and water. Wood from the mutant had more extractable colored material than did the normal wood, with significant amounts of coniferaldehyde and vanillin (19). Six cycles of acetone and water extractions removed most of the colored material. Klason lignin [R. D. Hatfield, H. G. Jung, J. Ralph, D. R. Buxton, P. J. Weimer, *J. Sci. Food Agric.* **65**, 51-58 (1994)] levels were 32% wt/wt for the *cad-n1* mutant and 31% for the normal wood. The ground wood was then ball milled and extracted with 96:4 dioxane:water. Saccharides and metal ions were removed using EDTA (7). The final yields of milled wood lignin were 12.5% of the lignin in normal pine and 17% in the *cad-n1* mutant; Klason lignin contents were 93% by weight for each, total carbohydrates were ~2% each and total uronosyls were 2-5%.
21. Vanillin is produced from coniferaldehyde by an aldol reaction that can occur at neutral pH [K. V. Sarkanen, C. H. Ludwig, *Lignins, Occurrence, Formation, Structure and Reactions* (WileyInterscience, New York, 1971)].
22. Long-range C–H correlation NMR experiments (not shown) establish that a correlated oxygenated aromatic carbon is within 3-bonds of the methoxyl protons and the aldehyde proton. These correlations are only possible from 2-methoxybenzaldehydes.
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24. NMR data for model compounds, dihydroconiferyl alcohol moiety sidechain resonances only. Model **8a**, 1-(4-hydroxy-3-methoxyphenyl)-2-[4-(3-hydroxy-propyl)-2-methoxy-phenoxy]propane-1,3-diol, the b-ether cross-product of coniferyl alcohol and dihydroconiferyl alcohol: nmr (acetone-d_6): δ_C/δ_H: 32.6/2.60 (α), 35.9/1.78 (β), 61.7/3.55 (γ). Model **8b**, 4-[3-hydroxymethyl-5-(3-hydroxypropyl)-7-methoxy-2,3-dihydrobenzofuran-2-yl]-2methoxyphenol, the phenylcoumaran (β-5) cross-product of coniferyl alcohol and dihydroconiferyl alcohol: nmr (acetone-d_6): δ_C/δ_H: 32.4/2.61 (α), 35.6/1.78 (β), 61.7/3.54 (γ). Model **8c**, di-(3-hydroxypropyl)-*trans*-6,7-dihydro-7-(4-hydroxy-3-methoxyphenyl)-4,9dimethoxydibenzo[e,g][1,4]dioxocin-6-yl methanol, the 5-5-coupled dimer of dihydroconiferyl alcohol then coupled 4-O-β to coniferyl alcohol to form a dibenzodioxocin: nmr (acetone-d_6): δ_C/δ_H: 32.81, 32.85/2.70, 2.74 (α), 35.58, 35.67/1.85, 1.89 (β), 61.78, 61.84/3.60 (γ).
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34. The authors are grateful to Fachuang Lu for providing analytical data via the new DFRC method (29) which verified the NMR findings and provided data on the high *p*-coumaryl alcohol component, to Yinsheng Zhang for preparing the synthetic lignin, to Sally Ralph, Larry Landucci and Frank Ludley for help in lignin preparation steps and model work on the coniferaldehyde components, and to John Grabber for his valuable input. NMR studies at 750 MHz for supporting data were carried out at the National Magnetic Resonance Facility at Madison. We thank George Askew (Baruch Experimental Forest, Clemson University, Georgetown, SC) for kindly providing plant material. The authors are grateful for partial funding from the USDA-NRI , Plant Growth and Development section (#94-02764 and #96-02587), grants from NIH (#GM45344-07), DOE (#DE-FG05-92ER20085) and the NCSU Forest Biotechnology Industrial Research Consortium.